Animal Feed Science and Technology 168 (2011) 206–222

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Evaluation of lignin contents in tropical forages using different analytical methods and their correlations with degradation of insoluble fiber

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ARTICLE INFO

Article history:
Received 21 October 2010
Received in revised form 11 April 2011
Accepted 4 May 2011

Keywords:
Acetyl bromide lignin
Indigestible neutral detergent fiber
Klason lignin
Potassium permanganate lignin
Sulfuric acid lignin

ABSTRACT

We compared the lignin contents of tropical forages by different analytical methods and evaluated their correlations with parameters related to the degradation of neutral detergent fiber (NDF). The lignin content was evaluated by five methods: cellulose solubilization in sulfuric acid [Lignin (sa)], oxidation with potassium permanganate [Lignin (pm)], the Klason lignin method (KL), solubilization in acetyl bromide from acid detergent fiber (ABLadf) and solubilization in acetyl bromide from the cell wall (ABLcw). Samples from ten grasses and ten legumes were used. The lignin content values obtained by gravimetric methods were also corrected for protein contamination, and the corrected values were referred to as Lignin (sa)p, Lignin (pm)p and KLp. The indigestible fraction of NDF (iNDF), the discrete lag (LAG) and the fractional rate of degradation (kd) of NDF were estimated using an *in vitro* assay. Correcting for protein resulted in reductions ($P<0.05$) in the lignin contents as measured by the Lignin (sa), Lignin (pm) and, especially, the KL methods. There was an interaction ($P<0.05$) of analytical method and forage group for lignin content. In general, LKp method provided the higher ($P<0.05$) lignin contents. The estimates of lignin content obtained by the Lignin (sa)p, Lignin (pm)p and LKp methods were associated ($P>0.05$) with all of the NDF degradation parameters. However, the strongest correlation coefficients for all methods evaluated were obtained with Lignin (pm)p and KLp. The lignin content estimated by the ABLcw method did not correlate ($P>0.05$) with any parameters of NDF degradation. There was a correlation ($P<0.05$) between the lignin content estimated by the ABLadf method and iNDF content. Nonetheless, this correlation was weaker than those found with gravimetric methods. From these results, we concluded that the gravimetric methods produce residues

Abbreviations: ABLadf, lignin determined by solubilization with acetyl bromide from the acid detergent fiber; ABLcw, lignin determined by solubilization with acetyl bromide from the cell wall matrix; CP, crude protein; DM, dry matter; iNDF, indigestible fraction of neutral detergent fiber; kd, fractional degradation rate of potentially degradable neutral detergent fiber; KL, lignin determined by Klason method; KLp, lignin determined by Klason method and corrected for protein; LAG, discrete lag for fiber degradation; Lignin (sa), lignin determined by solubilization of cellulose with sulfuric acid; Lignin (sa)p, lignin determined by solubilization of cellulose with sulfuric acid and corrected for protein; Lignin (pm), lignin determined by oxidation with potassium permanganate; Lignin (pm)p, lignin determined by oxidation with potassium permanganate and corrected for protein; NDF, neutral detergent fiber; pdNDF, potentially degradable fraction of neutral detergent fiber.

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0377-8401
doi:10.1016/j.anifeedsci.2011.05.001

that are contaminated by nitrogenous compounds. Adjustment for these contaminants is suggested, particularly for the KL method, to express lignin content with greater accuracy. The relationships between lignin content measurements and NDF degradation parameters can be better determined using KLp and Lignin (pm)p methods.

1. Introduction

The quantification of the nutritional value of ruminant feed requires studies that evaluate the fiber fraction of forages, which is of fundamental importance in the tropics and subtropics as it provides significant energy at low cost. The fiber fraction should occupy a crucial position in energy evaluation, as it is naturally more variable than the other chemical components, such as cell contents (Detmann et al., 2008).

Several factors have been studied in an attempt to clarify the use of forages by animals to optimize ruminant performance in the tropics. Factors associated with the composition of the cell wall have been found to be responsible for the lower forage intake and animal performance in the tropics. Of the components of the cell wall, lignin is considered the main limiting factor of the degradation of fibrous polysaccharides in the rumen (Van Soest, 1994).

Degradation of the cell wall requires an active microbial population that is capable of utilizing its components. Therefore, it depends on a complex interaction of microbial enzymes and substrate, which will determine the effectiveness of the degradation process (Detmann et al., 2009). Thus, the components of the cell wall that prevent the colonization and utilization of the fibrous fractions of feeds must be studied carefully not only as absolute chemical structures, but also in regard to their influence on the dynamic process of ruminal degradation.

In the majority of nutritional evaluations, the complexities of the biological mechanisms of digestion have been ignored because lignin content is correlated with the punctual digestibility of forages (Van Soest, 1963; Jung and Vogel, 1986; Jung and Varel, 1988; Fukushima and Hatifield, 2004). However, the process of substrate use in the gastrointestinal tract of the ruminant encompasses several more complex factors. Thus, the associations between the lignin content and variables related to the potential for and effectiveness of microbial degradation of insoluble fiber are important from a biological standpoint.

It is unclear which method produces most accurate estimates of lignin contents in tropical forages. Nevertheless, from a nutritional and functional standpoint, the methods for the quantification of lignin must be seen as a means to check the portion of the feed that corresponds to deleterious effects on the digestion of fibrous carbohydrates, which seems to be more important nutritionally when compared to evaluation of absolute chemical values.

No measure of forage quality can be fully correct, but the usefulness of the data resulting from efforts aimed at the improvement of forage quality is limited by how the material is characterized (Jung and Allen, 1995). Therefore, the measurement method that establishes the best relationship between lignin content and the fiber ruminal degradation of tropical forages remains undefined.

In this study, we aimed to evaluate lignin content in tropical forages by different analytical methods and to evaluate its relationship with neutral detergent fiber (NDF) degradation parameters in tropical grasses and legumes.

2. Materials and methods

2.1. Location and samples

The experiment was conducted at the Animal Nutrition Laboratory of the Animal Science Department of the Universidade Federal de Viçosa in Viçosa, Brazil, and at the Lignin Laboratory of the School of Veterinary Medicine and Animal Science of the Universidade de São Paulo in Pirassununga, Brazil.

Ten grasses, *Pennisetum purpureum*, *Brachiaria decumbens*, *Panicum repens*, *Brachiaria humidicula*, *Andropogon gayanus*, *Panicum maximum* cv. Aruana, *Panicum maximum* cv. Mombaça, *Brachiaria brizantha* cv. Xaraés, tifton 85 bermudagrass (*Cynodon* sp.) and *Panicum maximum* cv. Massai, and ten legumes, *Arachis pintoi*, *Medicago sativa*, *Leucaena leucocephala*, *Galactia striata*, *Dolichos lablab*, *Centrosema pubescens*, *Glycine weghtii*, *Gliricidia sepium*, *Stylosantes guianenensis* and *Cajanus cajan*, were evaluated. The forages were cultivated in 2-m × 4-m plots. All samples were cut at ground level in December 2008. The plants had approximately 45 days of regrowth.

The samples were oven-dried at 60 °C and processed in a knife mill (1-mm).

2.2. Laboratory analyses

2.2.1. Chemical composition of samples

The dry matter (DM, index no. 934.01), organic matter (index no. 942.05), crude protein (CP, index no. 954.01), and ether extract (index no. 920.39) contents of the samples were analyzed according to the methods of the AOAC (1990). For the NDF analysis, the samples were treated with a heat-stable alpha amylase without using sodium sulfite and corrected for residual ash (Mertens, 2002) and protein (Licitra et al., 1996) (Table 1).

Table 1
Chemical composition of forages.

Forage	DM[a,b]	OM[a,c]	CP[a,c]	EE[a,c]	aNDFom,p[a,c]	NFC[a,c,d]
Grasses						
P. purpureum	200.2	886.6	113.7	21.3	658.0	93.6
B. decumbens	305.8	920.6	78.5	36.9	710.3	94.9
P. repens	310.7	951.5	97.3	25.4	714.8	114.0
B. humidicola	290.2	930.9	70.2	23.1	723.0	114.5
A. gayanus	290.5	940.4	87.9	23.2	736.7	92.5
P. maximum cv Aruana	347.5	942.1	78.0	25.1	736.5	102.5
P. maximum cv Mombaça	294.6	905.6	103.9	12.8	690.4	98.8
B. brizantha cv Xaraés	272.1	933.1	86.8	23.8	719.8	102.7
Cynodon sp.	312.5	946.5	107.4	24.0	692.3	122.8
P. maximum cv Massai	321.0	932.9	72.6	21.5	799.5	39.0
Legumes						
Arachis pintoi	198.7	918.8	176.1	18.8	403.6	320.4
Medicago sativa	256.9	916.7	219.6	48.5	398.7	249.7
Leucena leucocephala	342.5	934.2	220.8	39.7	408.9	264.8
Galactia striata	332.9	924.8	198.7	26.9	407.1	292.1
Dolichos lablab	195.8	914.3	168.7	29.8	443.6	272.2
Centrozema pubescens	262.7	932.6	156.9	20.8	680.9	74.0
Glicine wightti	243.2	904.9	178.2	36.5	512.3	177.8
Gliricidia sepium	176.2	934.7	196.8	15.5	598.0	124.4
Stylosantes guianenensis	304.2	942.4	127.5	27.0	546.9	241.0
Cajanus cajan	293.8	949.2	169.8	29.0	692.5	57.9

[a] DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; aNDFom,p, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive for residual ash and protein; NFC, non-fibrous carbohydrates.
[b] g/kg.
[c] g/kg DM.
[d] NFC = OM − (CP + EE + aNDFom,p) (Detmann and Valadares Filho, 2010).

2.2.2. Lignin analyses

The lignin contents of forages were quantified by five different methods: cellulose solubilization by sulfuric acid after extraction with acid detergent [Lignin (sa)]; oxidation by potassium permanganate after extraction with acid detergent [Lignin (pm)]; the Klason lignin method (KL); the soluble lignin in acetyl bromide method after extraction with acid detergent (ABLadf) and the soluble lignin in acetyl bromide method using the cell wall residue (ABLcw).

To quantify the lignin content by the Lignin (sa) method, approximately 1 g of sample was conditioned in 120-mL polyethylene screw capped bottles, and 100 mL of acid detergent was added (Van Soest and Robertson, 1985). After sealing, the bottles were autoclaved at 105 °C for 1 h (Pell and Schofield, 1993). The acid detergent insoluble residue was retained by vacuum filtration in a filter crucible, washed sequentially with hot water and acetone, and oven-dried at 105 °C for 16 h. Afterward, the filter crucibles containing the residues were conditioned in polyethylene flasks and treated with 12 M sulfuric acid for 3 h as described by Van Soest and Robertson (1985). After that, the crucibles were subjected to vacuum filtration and washed with hot water to completely remove the acid. The material was oven-dried at 105 °C for 16 h and then weighed to obtain the mass of the residue composed of lignin and minerals. Then, the crucibles were transferred to a muffle furnace at 500 °C for 3 h. They were weighed again, and the mass of lignin was calculated by the weight loss after incineration.

To quantify the content of residual protein ($N \times 6.25$) associated with lignin, aliquots of residues obtained after treatment with sulfuric acid were evaluated according to the Kjeldahl method (AOAC, 1990).

The KL method for determining lignin content is based on the acid hydrolysis of the water-insoluble fraction (Theander and Westerlund, 1986). In this method, the material was not subjected to extraction with acid detergent. Approximately 250 mg of sample was conditioned in 120-mL polyethylene screw capped bottles. Three milliliters of 12 M sulfuric acid was added to the sample, which was stirred with a glass rod. The bottles were kept in a water bath at 30 °C for 30 min. Subsequently, 80 mL of distilled water was added to each pot, and the bottles were then sealed and autoclaved at 105 °C for 1 h. After autoclaving, while the contents were still warm, the insoluble material was quantitatively vacuum-transferred to filter crucibles and then washed with hot water and dried at 105 °C for 16 h. Subsequently, the crucibles were heated in the muffle furnace at 500 °C for 3 h. The weight after incineration was subtracted from the weight of the residue insoluble in sulfuric acid to calculate the lignin content. The residual protein was evaluated such described for Lignin (sa).

The Lignin (pm) method was performed by first obtaining the acid detergent insoluble residue as described for the Lignin (sa) method. Then, the filter crucibles containing the residue were placed in a polyethylene tray with a 2–3-cm layer of distilled water and sequentially extracted with a saturated $KMnO_4$ solution and a demineralizing solution as described by Van Soest and Wine (1968). After that, the residue was vacuum filtered, and washed with an ethanol solution (800 mL/L) and acetone. The crucibles containing the residue were oven-dried at 105 °C for 16 h. The lignin mass was calculated by the difference between the mass of the acid detergent insoluble residue and the residual mass after the treatment.

Subsequently, aliquots of the residues obtained after treatment with the permanganate and demineralizing solutions were evaluated according to the Kjeldahl method (AOAC, 1990). The protein content associated with lignin ($N \times 6.25$) was calculated by subtracting the protein in the residue obtained from the acid detergent insoluble protein (Licitra et al., 1996).

To isolate the cell wall for the evaluation of ABLcw, 10-g aliquots were conditioned in non-woven textile filter bags (100 g/m^2; 15 cm × 10 cm), which were conditioned in a Soxhlet extractor equipped with a heating blanket. The samples were subjected to sequential extraction with water, ethanol (960 mL/L), chloroform:methanol (2:1) and acetone. Approximately 250 mL of each solution were used and the extractions were supposed to be completed when the residual liquid was colorless. After extractions, the material was oven-dried at 60 °C for 72 h.

Material for ABLadf quantification was prepared in a similar manner to that described for the evaluation of Lignin (sa).

The residues obtained for the evaluation of ABLcw and ABLadf content were analyzed according to the recommendations of Fukushima and Kerley (2011).

A 100-mg sample of cell wall or acid detergent residue was weighed in a glass centrifuge tube with a Teflon cap. Then, 10 mL of a solution of acetyl bromide in acetic acid (250 mL/L) was added, and the sample was slowly homogenized. Subsequently, the tubes were kept in a water bath at 50 °C for 2 h. The contents were stirred every 30 min. A blank control was set up with the same series of tubes. After the tubes cooled down, the material was centrifuged at 2000 × *g* for 15 min. Then, 0.5-mL aliquots of the solutions, each containing around 5 mg of residues, were pipetted into test tubes containing 6.5 mL of glacial acetic acid and 2 mL of 0.3 M NaOH. The material was stirred. One milliliter of 0.5 M hydroxylamine hydrochloride solution was added, and the contents were further stirred.

The absorbance of the solution was read at 280 nm and converted into concentrations according to the equation suggested by Fukushima and Kerley (2011):

$$L = \frac{A - 0.0009}{23.077} \quad (1)$$

where L is the lignin concentration (mg/mL) and A is the absorbance.

The lignin contents determined by the ABLadf and ABLcp methods were considered free from protein contamination (Morrison, 1972); therefore, no correction was made.

2.3. Evaluation of NDF degradation

For the *in vitro* evaluation of the NDF degradation dynamics, aliquots of forage (350 mg of DM) were conditioned in 50-mL glass flasks. Subsequently, 28 mL of a buffer solution whose pH had previously been adjusted to 6.8 by flushing with CO_2 were added (McDougall, 1949).

The flasks were maintained in a climate-controlled room (39 °C) for prior hydration of the samples. During the hydration process, ruminal fluid was collected from a rumen-fistulated steer that was kept close to the incubation room. The animal was fed *ad libitum* with a mixed diet (80:20 forage:concentrate ratio) and complete mineral mixture.

The liquid was collected in the liquid:solid interface of the ruminal environment, filtered by a triple layer of cheesecloth, conditioned in an thermal container and immediately transported to the incubation room. Seven milliliters of ruminal inocula was added per flask. The incubation environment was immediately saturated with CO_2 and the flasks were quickly sealed. The flasks were maintained at 39 °C with orbital shaking (40 rpm). Gases arising from fermentation were removed every 3 h using needles.

Incubation times of 0, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h were evaluated. The incubation procedure was repeated four times, resulting in a total of four evaluations per incubation period for each forage sample. At the end of each incubation period, the flasks were removed from the climate-controlled room, and the contents were vacuum filtered in filter crucibles.

The crucibles were then conditioned in polyethylene flasks (120 mL) to which 50 mL of neutral detergent was added. After being sealed, the flasks were autoclaved at 105 °C for 1 h (Pell and Schofield, 1993). After that, residues were vacuum filtered, sequentially washed with hot water and acetone, and oven-dried at 105 °C for 16 h.

The NDF residues were subjected to adjustment by the non-linear logistic model described by Van Milgen et al. (1991) through the Gauss-Newton algorithm implemented in the PROC NLIN of SAS:

$$\mathrm{Rt} = \mathrm{pdNDF} \times (1 + \lambda \times t) \times \exp(-\lambda \times t) + \mathrm{iNDF} \quad (2)$$

where Rt is the non-degraded NDF residue at time "*t*" (g/100 g NDF); pdNDF is the potentially digestible NDF fraction (g/100 g NDF); iNDF is the indigestible NDF (g/100 g NDF); λ is the combined fractional rate of lag and degradation of pdNDF (h^{-1}); and t is the time (h).

Given that the λ parameter represents both the lag and degradation rates, the fractional rate of degradation rate of pdNDF (kd, h^{-1}) was estimated from λ using the gamma-2 distribution properties (Ellis et al., 1994):

$$\mathrm{kd} = 0.59635 \times \lambda \quad (3)$$

The estimates of discrete lag time were obtained according to Vieira et al. (1997):

$$LAG = \frac{R(0) - R(t_i)}{R'(t_i)} + t_i \quad (4)$$

where LAG is the discrete lag time (h); $R(0)$ is the non-degraded NDF residue at $t = 0$ (g/100 g NDF); $R(t_i)$ is the non-degraded NDF residue obtained at the point of inflection of the degradation curve (g/100 g NDF); $R'(t_i)$ is the derivative from the fitted degradation curve to the point of inflection (maximum rate of substrate degradation) (h^{-1}); and t_i is the time equivalent to the point of inflection on the degradation curve (h).

The t_i values were obtained according to Van Milgen et al. (1991):

$$t_i = \frac{1}{\lambda} \tag{5}$$

2.4. Statistical analyses

The values for crude lignin and lignin corrected for protein contaminants were compared independently for the Lignin (sa), Lignin (pm) and KL methods by adjusting the simple linear regression equation of corrected values (dependent variable) on crude values (independent variable); the statistical analysis was conducted under the null hypothesis:

$$H_0: \quad \beta_0 = 0 \quad \text{and} \quad \beta_1 = 1 \tag{6}$$

Corrected and crude lignin contents were considered to be similar when the null hypothesis was not rejected.

The lignin contents obtained by the different methods were directly compared between the different species groups (grasses or legumes) according to the model:

$$Y_{ijk} = \mu + G_i + S_{(i)j} + M_k + GM_{ik} + \varepsilon_{ijk} \tag{7}$$

where μ is the general constant; G_i is the effect of the species group i (grass or legume; fixed effect); $S_{(i)j}$ is the effect of species j nested within group i (random effect); M_k is the effect of the kth method of analysis (fixed effect); GM_{ik} is the interaction effect of the species group i and the method k; and ε_{ijk} is the random error.

The total number of observations used in the analysis of variance was 100, consisting of plant groups (2), species within groups (10) and lignin methods (5).

The relationships between the lignin contents obtained by the different methods and the characteristics of NDF degradation (kd, LAG and iNDF) were evaluated by linear regression using a dummy variable (Draper and Smith, 1966) according to the basic model:

$$Y_{ij} = \beta_0 + \beta_1 \times D + \beta_2 \times L_{ij} + \beta_3 \times (D \times L_{ij}) + e_{ij} \tag{8}$$

where Y_{ij} is the dependent variable observed in species j of group i; L_{ij} is the lignin content (g/kg NDF); D is the dummy variable corresponding to the group of species, with $D = 0$ for grasses and $D = 1$ for legumes; and e_{ij} is the random error.

The best model for the description of relationships was chosen via the backward regression method (Draper and Smith, 1966).

All statistical procedures were performed using SAS (*Statistical Analysis System*) (PROC MIXED and PROC NLIN) ($\alpha = 0.05$).

3. Results

Correcting for contaminant protein reduced the lignin contents estimated by the Lignin (sa), KL and Lignin (pm) methods, on average, by 0.8, 19.9 and 2.8 g/kg DM, respectively, in grasses. For legumes, the correction reduced the estimated lignin contents by 2.6, 38.9 and 7.9 g/kg DM, respectively (Table 2). There was higher protein contamination in the lignin contents evaluated gravimetrically in legumes than there was in grasses.

Considering both units of expression of lignin content (g/kg DM and g/kg NDF), the adjustment for protein contamination resulted in a reduction ($P<0.05$) of the lignin contents obtained by the Lignin (sa), KL and Lignin (pm) methods (Table 3).

Due to the reduction ($P<0.05$) in the lignin contents obtained by the Lignin (sa), KL and Lignin (pm) methods as a result of the corrections, we used the adjusted values, Lignin (sa)p, KLp and Lignin (pm)p, as the basis for further analysis and discussion. This decision was based on the fact that the nitrogenous compounds of the cell wall do not exert inhibitory effects on the degradation of fibrous carbohydrates, thus allowing a more accurate comparison with the contents evaluated by the ABLadf and ABLcw methods, which are considered to be free from protein contamination.

There was an interaction effect ($P<0.05$) of the analytical method and the forage group on lignin content considering both evaluated units (g/kg DM and g/kg NDF; Table 4). In general, KLp produces the higher lignin contents ($P<0.05$). The pattern of lignin contents was quite variable among methods and between forage groups (Table 4).

Despite the differences between the methods (Table 4), the Lignin (sa)p, KLp and Lignin (pm)p measurements were correlated with each other ($P<0.05$), with moderate to strong correlation coefficient estimates (Table 5). On the other hand, the measurements obtained by the spectrophotometric methods, ABLadf and ABLcw, were not correlated with each other ($P>0.05$) or with the measurements obtained by the gravimetric methods (Table 5).

Considering that the inhibitory effects of lignin on degradation are only observed in the fibrous fraction, the relationships with the NDF degradation parameters were interpreted only in terms of the unit g/kg NDF. Similarly to what was previously described, lignin content obtained by gravimetric methods and adjusted for protein contamination was considered.

The estimates of lignin content obtained by the Lignin (sa)p and Lignin (pm)p methods were related ($P<0.05$) to the iNDF content as well as to kd and LAG (Table 6 and Figs. 1 and 2). However, for both methods, there were no differences in behavior

Table 2
Descriptive statistics for the evaluated variables.

Item[a]	Grasses		Legumes	
	Mean	SD	Mean	SD
g/kg DM				
NDFap	568.5	42.3	412.4	62.3
Lignin (sa)	63.4	11.1	92.3	21.5
Lignin (as)p	62.6	10.7	89.7	18.6
KL	154.2	20.9	171.3	56.2
KLp	134.3	18.6	132.4	47.1
Lignin (pm)	83.9	7.0	116.8	16.4
Lignin (PM)p	81.1	6.6	108.9	17.4
ABLcw	79.7	16.7	53.1	49.9
ABLadf	46.9	11.1	41.6	6.6
g/kg NDF				
Lignin (sa)	111.5	18.3	224.1	43.3
Lignin (as)p	110.3	17.7	217.7	37.6
KL	271.8	38.9	413.8	122.3
KLp	236.8	35.1	319.3	101.8
Lignin (pm)	147.8	11.1	286.1	37.9
Lignin (PM)p	142.8	10.7	266.6	40.1
ABLcw	141.4	34.1	131.8	25.0
ABLadf	82.8	19.9	101.3	9.8
Degradation parameters				
iNDF	369.3	48.7	510.4	84.7
LAG	3.43	0.47	4.71	1.20
kd	0.0498	0.0070	0.0376	0.0088

[a] NDFap, neutral detergent fiber corrected for ash and protein; Lignin (sa), lignin determined by solubilization of cellulose with sulfuric acid; Lignin (sa)p, lignin determined by solubilization of cellulose with sulfuric acid and correctd for protein; KL, Klason lignin; KLp, Klason lignin corrected for protein; Lignin (pm), lignin determined by oxidation with potassium permanganate; Lignin (pm)p, lignin determined by oxidation with potassium permanganate and corrected for protein; ABLadf, lignin determined by solubilization with acetyl bromide from the acid detergent fiber; ABLcw, lignin determined by solubilization with acetyl bromide from the cell wall matrix; iNDF, indigestible fraction of neutral detergent fiber (g/kg NDF); LAG, discrete lag (h); kd, fractional degradation rate of potentially degradable neutral detergent fiber (h^{-1}).

Table 3
Estimates of regression parameters of relationship between crude (X) and protein corrected (Y) lignin contents according to different gravimetric methods.

Methods[a]	Regression parameters				
	Intercept	Slope	r^2	s_{xy}	P-value[b]
g/kg DM					
Lignin (sa)	4.8238	0.9163	0.9941	1.61	<0.0001
KL	5.4141	0.7859	0.9010	11.34	<0.0001
Lignin (pm)	3.5644	0.9109	0.9772	2.98	<0.0001
g/kg NDF					
Lignin (sa)	6.7810	0.9370	0.9960	4.06	<0.0001
KL	31.3182	0.7196	0.9345	22.44	<0.0001
Lignin (pm)	6.5452	0.9135	0.9920	6.42	<0.0001

[a] Lignin (sa), lignin determined by solubilization of cellulose with sulfuric acid; KL, Klason lignin; Lignin (pm), lignin determined by oxidation with potassium permanganate.
[b] H_0: $\beta_0 = 0$ and $\beta_1 = 1$ (Eq. (6)).

Table 4
Evaluation of interaction effect of analytical method and forage groups on average lignin contents.

Forage	Method[a]					
	Lignin (as)p	KLp	Lignin (pm)p	ABLcw	ABLadf	SEM
g/kg DM[b]						
Grasses	62.6Bc	134.3Aa	81.1Bb	79.7Ab	46.9Ad	6.24
Legumes	89.7Ac	132.4Aa	108.9Ab	53.1Bd	41.6Ad	
g/kg NDF[b]						
Grasses	110.3Bc	236.8Ba	142.8Bb	141.4Ab	82.8Ac	13.18
Legumes	217.7Ac	319.3Aa	266.6Ab	131.8Ad	101.3Ad	

[a] Lignin (sa)p, lignin determined by solubilization of cellulose with sulfuric acid and corrected for protein; KLp, Klason lignin corrected for protein; Lignin (pm)p, lignin determined by oxidation with potassium permanganate and corrected for protein; ABLadf, lignin determined by solubilization with acetyl bromide from the acid detergent fiber; ABLcw, lignin determined by solubilization with acetyl bromide from the cell wall matrix.
[b] Means in the column followed by different capital letters or in the row followed by different lower case letters are different according to Fishers' LSD (P<0.05).

Table 5
Partial correlations between lignin contents (g/kg NDF) obtained by different methods.

Method	Method[a,b,c]			
	Lignin (as)p	KLp	Lignin (pm)p	ABLcw
KLp	0.7397 (0.0003)	–		
Lignin (pm)p	0.5954 (0.0072)	0.6545 (0.0024)	–	
ABLcw	−0.0046 (0.9850)	0.0743 (0.7625)	0.3760 (0.1126)	–
ABLadf	0.2034 (0.4036)	0.1044 (0.6705)	0.1550 (0.5263)	−0.0103 (0.9667)

[a] Lignin (sa)p, lignin determined by solubilization of cellulose with sulfuric acid and corrected for protein; KLp, Klason lignin corrected for protein; Lignin (pm)p, lignin determined by oxidation with potassium permanganate and corrected for protein; ABLadf, lignin determined by solubilization with acetyl bromide from the acid detergent.
[b] The correlation estimates are adjusted regarding forage groups effect.
[c] The values between parenthesis correspond to descriptive level of probability for type I error associated with H_0: $\rho = 0$.

between grasses and legumes in terms of the intercept or the slope of the fitted line (P>0.05). Lignin content estimates made using the Lignin (pm)p method were more strongly correlated with the NDF degradation parameters than were those made by the Lignin (sa)p method (Table 6).

The lignin contents estimated by KLp were associated (P<0.05) with all NDF degradation parameters (Table 6). Nevertheless, unlike the Lignin (sa)p and Lignin (pm)p methods, there was a difference between grasses and legumes in terms of the intercept of the fitted line (P<0.05); higher iNDF and LAG values and lower kd values were observed for legumes, regardless of the lignin concentration (Fig. 3). The inclusion of an additional parameter (intercept adjustment by using the dummy variable; Eq. (8)), based on which peculiarities of the forage groups were considered, resulted in the KLp method having

Table 6
Estimates of regression parameters for relationship between parameters of neutral detergent fiber degradation and lignin contents obtained by different methods.

Item[a]	Method[b,c]				
	Lignin (sa)p	KLp	Lignin (pm)p	ABLcw	ABLadf
iNDF (g/kg NDF)					
β_0	242.3	255.9	187.7	369.3	179.8
β_1	–	111.3	–	141.1	–
β_2	1.2292	0.4790	1.2817	–	2.8263
β_3	–	–	–	–	–
P-Value	0.0001	<0.0001	0.0004	0.0002	0.0208
r	0.7698	0.8534	0.8237	–	0.5209
r^d	0.7541	0.8333	0.8121	–	0.4779
s_{xy}	67.20	58.68	71.56	69.29	87.27
kd (h^{-1})					
β_0	0.0590	0.0605	0.0637	0.0499	0.0499
β_1	–	−0.0067	–	−0.0106	−0.0106
β_2	−0.000087	−0.000045	−0.000095	–	–
β_3	–	–	–	–	–
P-Value	0.0046	0.0030	0.0010	0.0056	0.0056
r	−0.6200	−0.7179	−0.6934	–	–
r^d	−0.5901	−0.6744	−0.6710	–	–
s_{xy}	0.0072	0.0066	0.0066	0.0073	0.0073
LAG (h)					
β_0	2.60	2.45	2.16	3.43	3.43
β_1	–	0.63	–	0.98	0.98
β_2	0.0080	0.0041	0.0088	–	–
β_3	–	–	–	–	–
P-Value	0.0038	0.0019	0.0007	0.0040	0.0040
r	0.6301	0.7372	0.7077	–	–
r^d	0.6013	0.6974	0.6867	–	–
s_{xy}	0.64	0.58	0.58	0.64	0.64

[a] See more details about parameters in Eq. (8).
[b] Lignin (sa)p, lignin determined by solubilization of cellulose with sulphuric acid and corrected for protein; KLp, Klason lignin corrected for protein; Lignin (pm)p, lignin determined by oxidation with potassium permanganate and corrected for protein; ABLadf, lignin determined by solubilization with acetyl bromide from the acid detergent fiber; ABLcw, lignin determined by solubilization with acetyl bromide from the cell wall matrix.
[c] iNDF, indigestible fraction of neutral detergent fiber; LAG, discrete lag; kd, fractional degradation rate of potentially degradable neutral detergent fiber.
[d] Correlations adjusted for the number of parameters in the fitted model (Draper and Smith, 1966).

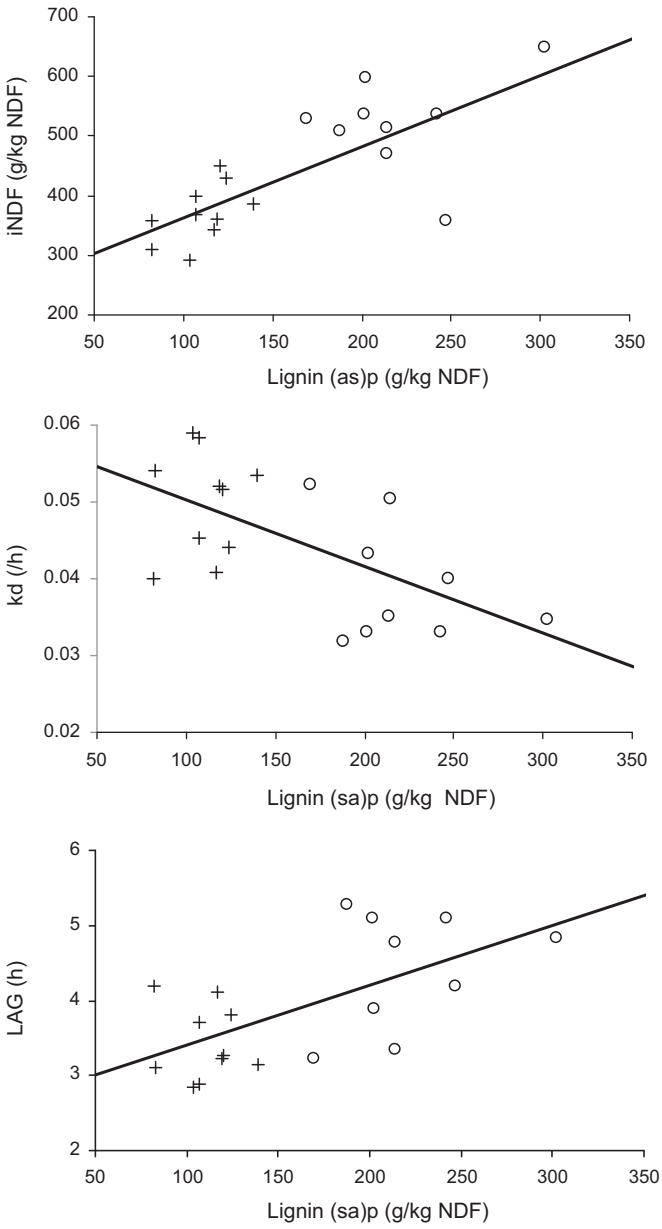

Fig. 1. Relationship between lignin obtained by solubilization with sulfuric acid and corrected for protein [Lignin (sa)p] and the indigestible neutral detergent fiber (iNDF), the fractional degradation rate of potentially degradable neutral detergent fiber (kd) and the discrete lag (LAG) (+ = grasses; ○ = legumes).

stronger correlation coefficients than all of the other methods evaluated. However, when the correlation coefficients were adjusted for the number of parameters in the model, they were similar to those obtained with the Lignin (pm)p method (Table 6).

The lignin contents estimated by the ABLcw method showed no functional relationship ($P>0.05$) to any of the parameters of NDF degradation dynamics. For all of these parameters, only the average difference between the forage groups was obtained (Fig. 4).

There was an association ($P<0.05$) between the lignin contents estimated by the ABLadf method and the iNDF contents; however, there were no differences between grasses and legumes ($P>0.05$). Nevertheless, the correlation coefficient was weaker than those found for the Lignin (sa)p, KLp and Lignin (pm)p methods (Table 6). In addition, as for the ABLcw method, no functional relationships ($P>0.05$) were found between lignin content and the kd and LAG parameters (Table 6), in which only an average difference between the groups of forages was detected (Fig. 5).

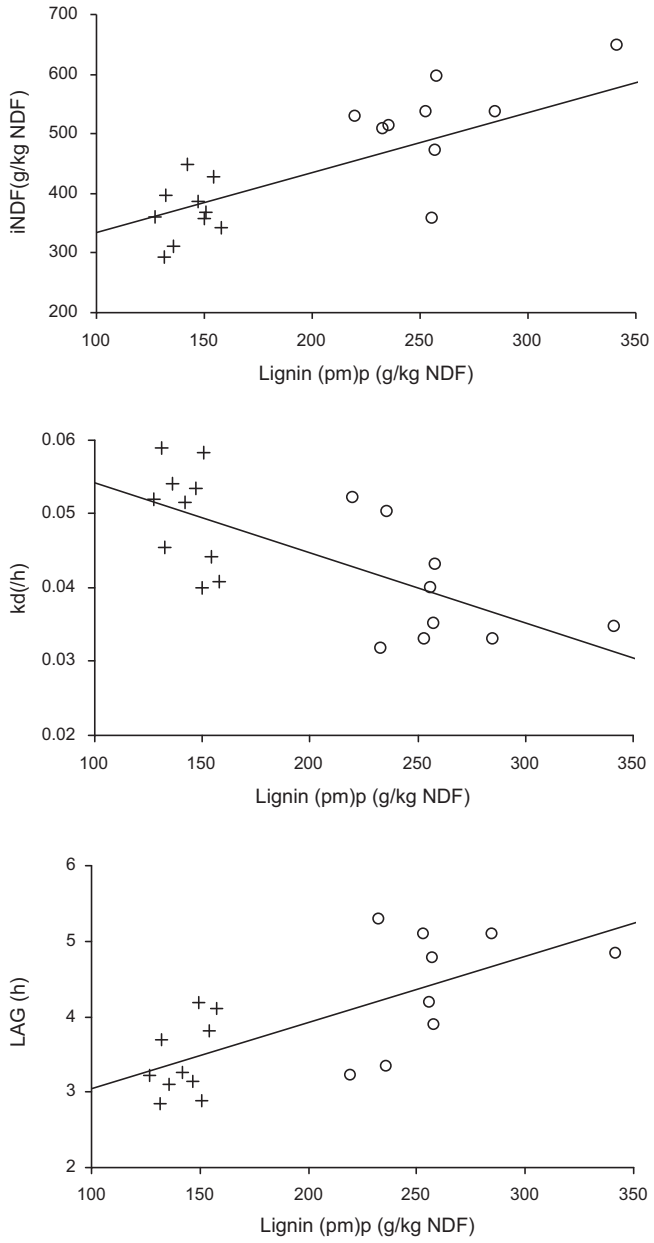

Fig. 2. Relationship between lignin obtained by oxidation with potassium permanganate and corrected for protein [Lignin (pm)p] and the indigestible neutral detergent fiber (iNDF), the fractional degradation rate of potentially degradable neutral detergent fiber (kd) and the discrete lag (LAG) (+ = grasses; ○ = legumes).

4. Discussion

This study showed that protein contamination significantly affected the estimates of lignin content by all gravimetric methods. However, this contamination was more evident for the KL method than for the Lignin (sa) and Lignin (pm) methods (Tables 2 and 3).

Among the gravimetric methods of lignin analysis, KL has the highest protein contamination (Fukushima and Hatifield, 2001), which constitutes one of the main biases in the estimates obtained by this method. KL was initially developed for the evaluation of lignin content in wood. In this type of material, protein contamination problems are not expected due to the low protein concentration in the samples (Whitehead and Quicke, 1964; Theander and Westerlund, 1986). However, the use of KL with no adjustment to quantify the lignin content in forages becomes complicated because of the significant presence of nitrogenous compounds in the insoluble residue that could be incorrectly classified as lignin (Van Soest, 1994).

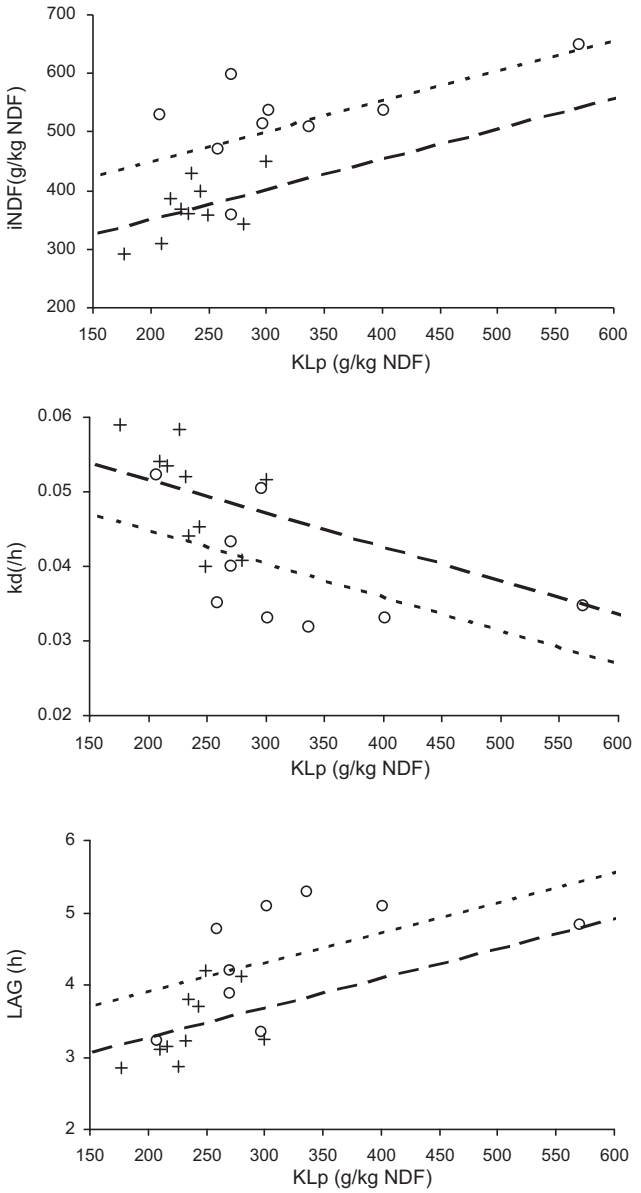

Fig. 3. Relationship between lignin obtained by Klason method and corrected for protein (KLp) and the indigestible neutral detergent fiber (iNDF), the fractional degradation rate of potentially degradable neutral detergent fiber (kd) and the discrete lag (LAG) (+ = grasses; ◯ = legumes).

The nitrogenous compounds associated with lignin residue can originate from four potential sources: nitrogen that is naturally associated with the cell wall, nitrogen attached to Maillard artifacts, nitrogen linked to tannins, and keratins of animal origin (Van Soest, 1994). The last source is not applicable to the results of this study.

It is presumed that legumes show higher protein contamination than do grasses (Fukushima and Hatifield, 2001), as found in this study (Table 2). Although legumes naturally have higher protein contents than grasses do, the level of CP in a sample was not correlated with the degree of protein contamination in lignin residues (Hatifield et al., 1994). However, as previously shown, the correction of the KL values for protein contamination reduced the average estimated lignin contents of legumes by 38.9 g/kg, compared to 19.9 g/kg DM for grasses (Table 2). At least part of this greater contamination in legumes can be traced to their greater tannin content, which would result in the formation of insoluble complexes with the protein components of forages (Van Soest, 1994).

The analysis of lignin content is laborious and time consuming, and it can produce residues consisting partly of artifacts that overestimate lignin content (Van Soest, 1963). Part of the protein contamination related to lignin could be attributed to the formation of artifacts by the Maillard reaction. However, their formation occurs mainly during drying at temperatures of at least 65 °C, which were avoided in this study (samples were dried at 60 °C). The use of adequate temperatures and

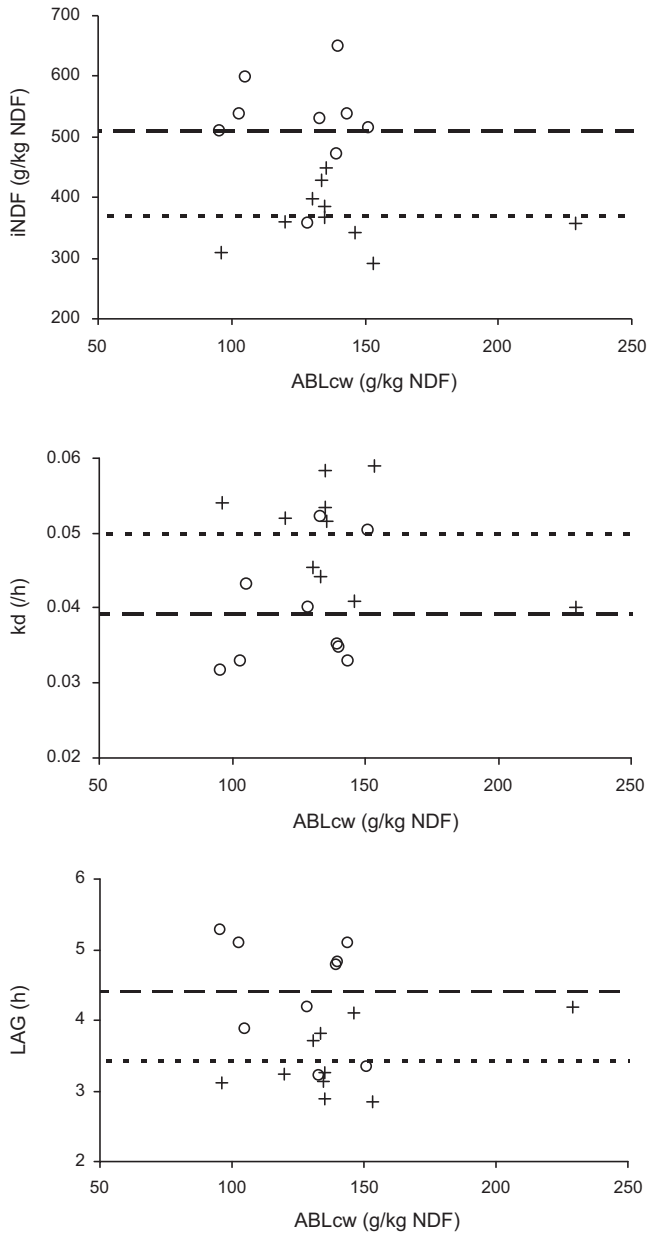

Fig. 4. Relationship between lignin obtained by solubilization with acetyl bromide from cell wall (ABLcw) and the indigestible neutral detergent fiber (iNDF), the fractional degradation rate of potentially degradable neutral detergent fiber (kd) and the discrete lag (LAG) (+ = grasses; ○ = legumes).

ventilated ovens reduces the formation of these artifacts by accelerating the removal of humidity from the material, which is necessary for non-enzymatic reactions to occur (Van Soest, 1994).

In addition, the use of high temperatures during the extraction process could be related to the formation of nitrogen-containing artifacts. However, the Maillard reaction is not favored under acid conditions (Eskin et al., 1971); there is no evidence that insoluble artifacts form between the cellular protein and the products of cell wall carbohydrate hydrolysis (Hatifield et al., 1994).

Therefore, despite the possibility of protein–tannin complex formation in legumes, most of the protein contamination associated with KL appears to be related to retention of nitrogenous compounds naturally present in the cell wall in insoluble residues.

The cell wall contains proteins that have a structural role in the matrix, which may have cross-links with lignin (Whitmore, 1982). However, it is not clear whether the nitrogenous compounds present in the insoluble residue of KL represent intact proteins, protein fragments, modified proteins or nucleic acids (Hatifield et al., 1994). Nonetheless, regardless of the origin of

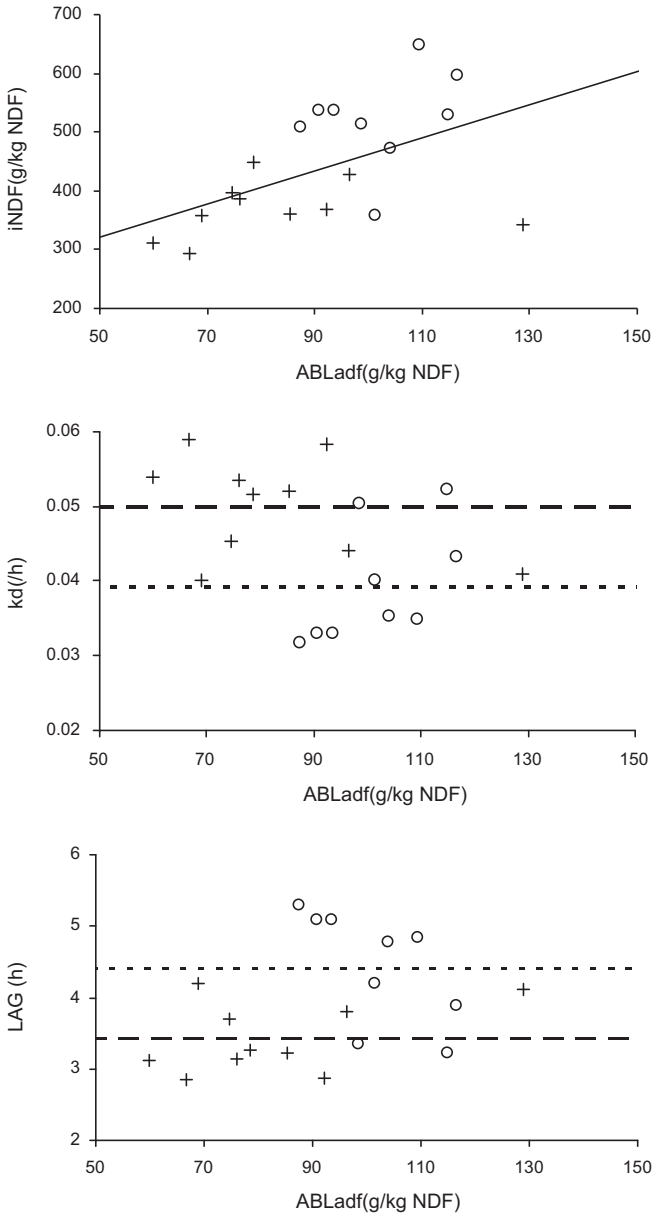

Fig. 5. Relationship between lignin obtained by solubilization with acetyl bromide from acid detergent fiber (ABLadf) and the indigestible neutral detergent fiber (iNDF), the fractional degradation rate of potentially degradable neutral detergent fiber (kd) and the discrete lag (LAG) (+ = grasses; ◯ = legumes).

the nitrogenous compounds, the main problem in the chemical fractionation of the fibrous tissue of the plant is the efficient separation of proteins and lignin (Van Soest, 1963).

The main analytical difference between the KL and Lignin (sa) methods is the sequence in which the different concentrations of sulfuric acid and extraction temperatures are used, which causes different effects on the hydrolysis of polysaccharides (Hatifield et al., 1994). However, it must be noted that cationic detergents (e.g., cetyltrimethylammonium bromide, CTAB) are not used in the KL method as an accessory in the removal of materials to be solubilized, which allows the cleaning of the material that will be subjected to acid hydrolysis. Thus, the acid detergent solution (20 g/L CTAB in 0.5 M H_2SO_4) is responsible for obtaining a residue that is nearly free from protein interference (Van Soest and Robertson, 1985), promoting solubilization of much of the protein associated with the cell wall (Van Soest, 1994).

Because of the previous extraction with acid detergent, Lignin (pm) showed lower levels of protein contamination than KL but slightly higher protein contamination levels than were observed with Lignin (sa) (Table 2). This finding seems to be justified by the fact that some proteins that are not removed by acid detergent can react with the permanganate and be quantified as lignin (Van Soest and Wine, 1968).

According to Van Soest (1994), even when lignin analyses are performed carefully, contamination will still exist regardless of the gravimetric method used, which corroborates the results found in this study (Table 3). Therefore, to correctly express the concentrations of lignin obtained by gravimetric methods, especially KL, protein contamination must be corrected for (Henriques et al., 2007).

In this context, the methods were directly compared, emphasizing the correction for protein contamination in the residues measured gravimetrically and thus avoiding confusion because there are differences between these methods in terms of contamination intensity (Table 2). The results obtained spectrophotometrically are considered free from this type of contaminant (Fukushima and Kerley, 2011).

Lignin contents in forage samples vary according to the method of chemical isolation of the polyphenolic molecule. The physical properties of lignin are generally changed by strong acids, which promote polymerization and additional condensation and can convert part of the originally soluble material into insoluble products (Van Soest, 1994).

The difference in lignin contents measured in the same sample by different methods may result from differences in the mechanisms of action of the reagents. This finding implies that different analytical procedures provide different estimates of lignin content (Fukushima and Dehority, 2000).

The methods for lignin analysis can be divided into three main categories: gravimetric methods like Lignin (sa) and KL that remove cell wall constituents but leave lignin behind; gravimetric methods like Lignin (pm) that oxidize lignin from the cell wall matrix; and solubilization methods employing spectrophotometric quantification, such as ABLadf and ABLcw.

However, in addition to the chemical peculiarities of each method, differences in the results can also be obtained based on differences in sample preparation steps. The use of acid detergent, which reduces protein contamination (Van Soest, 1994), also generally underestimates the lignin contents of forages, especially grasses, as a result of the partial solubilization of phenolic compounds (Lowry et al., 1994; Fukushima and Hatifield, 2001; Fukushima et al., 2009). In general, these aspects support the finding of lower lignin values with Lignin (sa)p and Lignin (pm)p than with KLp and with ABLadf in comparison to ABLcw (Table 4).

Although Lignin (pm)p and Lignin (sa)p are both gravimetric methods that are preceded by acid detergent extraction, higher lignin content estimates were found with Lignin (pm)p than with Lignin (sa)p (Table 4). According to Van Soest and Wine (1968), the Lignin (pm):Lignin (sa) ratio is approximately 1.2:1. In agreement with their findings, we obtained ratios of 1.30:1 and 1.23:1 for grasses and legumes, respectively (based on the g/kg NDF values in Table 4).

The evaluation of lignin contents by oxidation in potassium permanganate can be affected by some sample components, such as phenols and other unsaturated substances, including tannins and pigments, that are not completely removed during acid detergent extraction. These substances react with the permanganate solution and are thus counted as lignin, especially in immature grasses (Van Soest and Wine, 1968), increasing the total lignin content of the sample. This could justify, at least partly, the higher estimates given by the Lignin (pm)p method compared to the Lignin (sa)p method (Table 4).

Generally, the main limitation in the use of the KL method is protein contamination, which generates a positive bias in the lignin content estimates (Van Soest and Robertson, 1985; Kondo et al., 1987). However, after correction, the high values estimated by KLp cannot be attributed to protein contamination.

Although the KL method was developed to extract lignin from wood (Van Soest, 1994), it can be used to quantify lignin from feeds used for ruminant nutrition. However, even after modifications in which direct heating was incorporated (Theander and Westerlund, 1986), the method continues to be applied to intact samples. Therefore, it is speculated that sulfuric acid can solubilize part of the hemi-cellulose contained in the cell wall, which precipitates with the dilution of acid with water, leading to its quantification as lignin (Van Soest, 1967). Compounds such as D-galacturonic acid, D-glucuronic acid and D-xylose can be converted into aromatic compounds in heated and slightly acidified aqueous media (Popoff and Theander, 1976), similar to the second stage of the KL method.

Nevertheless, Hatifield et al. (1994) found that the contamination by carbohydrates in the lignin obtained by the KL method could be considered small enough not to contribute significantly to the residue. On the other hand, these authors found that the syringyl:guaiacyl ratios of lignin residues obtained by the Lignin (sa) and KL methods were similar. Therefore, there seems to be no significant contamination with phenolic compounds formed from carbohydrates that could alter the syringyl:guaiacyl ratio.

Thus, the difference between the KLp and Lignin (sa)p methods and, consequently, between the KLp and Lignin (pm)p methods is in the solubilization of some of the lignin by acid detergent (Hatifield et al., 1994; Lowry et al., 1994). In this case, the dissolution of the hemicellulose matrix by acid detergent would leave part of the lignin without the support given by hemicellulose, allowing its solubilization (Lowry et al., 1994). In support of this idea, there is evidence that KLp provides more accurate estimates of the total lignin content in forage samples than does Lignin (sa)p, especially in grasses (Kondo et al., 1987; Hatifield et al., 1994; Jung et al., 1997).

The acetyl bromide-soluble lignin method, a spectrophotometric method developed to quantify lignin contents in small wood samples, is based on measurement of the sample's absorbance at 280 nm after solubilization in a solution of acetyl bromide in acetic acid (Johnson et al., 1961). This method was modified for use in forage samples, which have a considerable amount of protein that significantly interferes with the measurement of lignin contents (Morrison, 1972). Fukushima et al. (2009) found, however, that the effluent from the acid detergent solution in several grasses showed peaks of absorbance similar to those of lignin retained in the acid detergent insoluble residue. Hence, the lignin content in acetyl bromide was evaluated based on the cell wall, providing more credible estimates of the lignin content of forages (Iiyama and Wallis, 1990; Fukushima and Dehority, 2000; Fukushima and Hatifield, 2001, 2004; Fukushima and Savioli, 2001; Chang et al., 2008).

The isolation of the cell wall by sequential washing with water, ethanol, chloroform and acetone aims to credibly represent the amount of polysaccharides in the cell wall (Fukushima and Hatifield, 2004). Therefore, this preparation of the cell wall would be characterized by higher lignin values when compared to other methods of isolation of cell wall components, such as ADF. However, if the non-lignin components and other phenolic compounds, like tannins, are not removed during the cell wall preparation step, they can be dissolved in the acetyl bromide solution, leading to interference during sample reading (Morrison, 1972).

The lack of standards for spectrophotometer calibration is the main limiting factor for the routine use of the method (Savioli et al., 2000). Therefore, in a recent study, lignin extracted with acetyl bromide and subsequently corrected for carbohydrate, ash, water and protein contents were used to make a universal standard curve in an attempt to shorten the analytical procedure to sample digestion and spectrophotometric reading only (Fukushima and Kerley, 2011). This universal standard curve was used in this study.

The lignin contents obtained for grasses by the ABLcw method were greater than were those obtained by the Lignin (sa)p method (Table 4). This result is expected given the partial solubilization of lignin during the acid detergent extraction step of the Lignin (sa)p method, corroborating the results of other authors (Fukushima and Dehority, 2000; Fukushima and Savioli, 2001; Fukushima and Hatifield, 2004).

For legumes, however, the lignin contents obtained with the ABLadf and ABLcw methods were similar, whereas ABLcw produced lower estimates than Lignin (sa)p. This pattern of results is different from that observed for grasses (Table 4) and contradicts the proposed explanations for the differences between the methods presented previously.

Generally, lignin contents obtained by the ABLcw method were higher than those obtained with the Lignin (sa)p method (Fukushima and Dehority, 2000). However, differing results can be found in the literature (Fukushima and Savioli, 2001).

One of the difficulties of quantifying lignin in acetyl bromide lies in obtaining a satisfactory spectrophotometric standard for lignin (Savioli et al., 2000; Hatfield and Fukushima, 2005). Generally, evaluations of lignin in acetyl bromide using grass samples have been more intensely studied compared to evaluations of legume samples. The set of samples used by Fukushima and Kerley (2011) for a universal standard curve had only 3 legumes in a set of samples from 14 species that also included grasses, tree species and 3 commercial lignins. Considering that the chemical compositions of lignin from grasses and legumes differ (Van Soest, 1994), the apparent distortions found in this study may indicate that the prediction efficiency of the universal standard curve (Eq. (1)), although apparently high for grasses, is low for legumes.

Simply knowing the NDF content of a feed is not enough to generate information about the potential for insoluble fiber to be utilized in the gastrointestinal tract in ruminants. In other words, two feeds can have similar NDF contents but different potentials for utilization. This finding limits inferences based solely on NDF content from a nutritional standpoint. Knowledge of the degradation dynamics of different NDF sources in the ruminal ecosystem is necessary to increase the knowledge about effective digestibility and the potential for the implementation of physically restrictive effects on voluntary intake (Detmann et al., 2008, 2009).

However, infrastructure and time limitations constrain data collection to characterize NDF degradation dynamics. Thus, it is crucial to find a characteristic that is capable of generating information that quickly determines, with relative precision, the capacities of different NDF sources to be utilized by ruminants.

Because it is indigestible and reduces the potentially degradable fibrous fraction (Traxler et al., 1998), lignin is primarily responsible for the limitation of degradation of fibrous forage components (Van Soest, 1994). The laboratory estimate of its concentration is fast and requires less infrastructure than do *in situ* or *in vitro* studies of NDF degradation dynamics. Thus, it is necessary to identify which of the analytical methods best discriminates forages with regard to NDF ruminal degradation aspects. According to Lowry et al. (1994), rumen fermentation characteristics that define the cell wall fraction that is deleterious to microorganisms are more important to the evaluation of feeds for ruminants than is defining exact chemical fractions.

Generally, the Lignin (sa)p and Lignin (pm)p estimates were similar in terms of their relationships with NDF degradation parameters (Table 6), showing biologically coherent results considering the positive associations between lignin content and iNDF and LAG contents and the negative associations between lignin and kd (Table 6 and Figs. 1 and 2). However, even though they are interrelated (Table 5), stronger correlations were observed with the Lignin (pm)p method than with Lignin (sa)p (Table 6). These results confirm and extend those obtained by Traxler et al. (1998) and Clipes (2007), who observed greater accuracy of iNDF prediction from Lignin (pm) in comparison to Lignin (sa) estimates in grasses and legumes.

As previously discussed, the role of sulfuric acid in the Lignin (sa) method is to oxidize the cellulosic components of the plant cell wall after extraction with acid detergent (Van Soest and Robertson, 1985), maintaining the phenolic components as residue; the role of potassium permanganate is to solubilize the phenolic compounds on the cell wall, also after treatment with acid detergent, producing a residue in which the cellulosic compounds are concentrated.

Although the Lignin (sa) and Lignin (pm) methods may seem perfectly complementary, problems lie in the exact definitions of the limits of action of each of the reagents (Van Soest, 1994), causing the lignin content estimates obtained by the two methods to differ, as seen in this study (Table 4). The greatest difference between the actions of sulfuric acid and of potassium permanganate can be seen in the boundary regions between cellulosic and phenolic compounds, a region in which there is greater inhibitory action of lignin on microbial degradation (Traxler et al., 1998; Clipes, 2007).

Therefore, the different actions of the above-mentioned reagents can lead to qualitative differentiations in the gravimetric estimates of the lignin contents of feeds. That is, the inhibitory action of the phenolic compounds retained as lignin and their extraction peculiarities in the areas adjacent to cell wall carbohydrates could have different associations with the insoluble

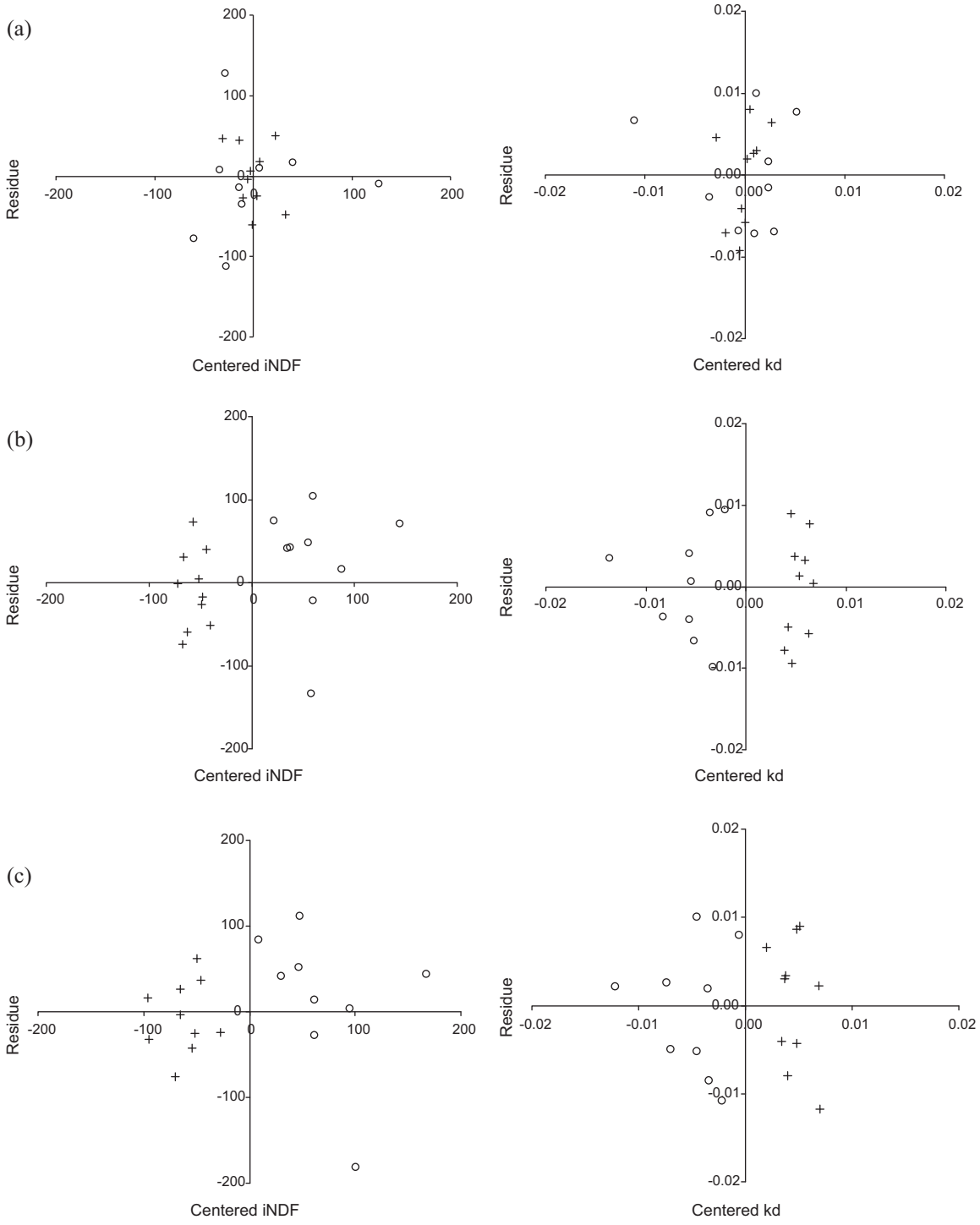

Fig. 6. Ordinary residual plots against the centered predicted values of iNDF (g/kg NDF) and kd (h^{-1}) [(a) Klason lignin corrected for protein; (b) lignin determined by oxidation with potassium permanganate and corrected for protein; (c) lignin determined by solubilization of cellulose with sulfuric acid and corrected for protein].

NDF fraction (Clipes, 2007) such that the lignin contents determined by Lignin (pm)p have a better association with the insoluble NDF fraction than do those determined by Lignin (sa)p (Table 6).

The differences between grasses and legumes indicated by Lignin (sa)p and Lignin (pm)p are only attributed to the lignin concentrations of the samples because no specific parameter for their differentiation was significant (Table 6).

On the other hand, the relationships between the NDF degradation parameters and the KLp contents considered, in addition to the differences in sample concentrations, a parameter related to the differentiation of the intercept of the function for grasses and legumes (Table 6 and Fig. 3). This additional discrimination in relation to Lignin (sa)p seems to be associated with the loss of the soluble lignin fraction in acid detergent, as previously discussed. In this context, the inclusion of an additional parameter in the function provided KLp with stronger correlations in relation to Lignin (sa)p, even considering the adjustment for the number of parameters of the model (Table 6). These results contradict those obtained by Jung et al. (1997), who found similar correlations between KL and Lignin (sa) and the *in vitro* and *in vivo* digestibility of NDF.

Considering the correlation coefficients adjusted for the number of parameters in the model, the Lignin (pm)p and KLp estimates were equally strongly associated with the NDF degradation parameters (Table 6). These methods showed higher lignin contents than Lignin (sa)p did (Table 4). In this context, the higher lignin contents estimated by the KLp and Lignin (pm)p methods may contain elements that have a significant role in the degradation of insoluble fiber, which would not be quantified in Lignin (sa)p, thus justifying the weaker correlations observed with this method (Table 6).

The residual evaluation of gravimetric methods does not reveal patterns that give evidence of model under-specification or heterogeneous variance and there were no systematic trend ($P>0.05$) of ordinary residues ($P>0.05$; Fig. 6). The residual plot was slightly more homogeneous for KLp compared to Lignin (sa)p and Lignin (pm)p, which seems to reflect the model discrimination with regard grasses and legumes (Table 6). Actually, the dataset evaluated in this work would not be considered completely adequate to suggest an accurate model to predict NDF degradation parameters. However, the evidences presented in Table 6 and Fig. 6 indicate that KLp should be considered when further models will be adjusted to estimate such characteristics from lignin contents.

Generally, with the exception of the relationship between ABLadf and iNDF, there were no associations between the lignin contents obtained by the spectrophotometric methods and the NDF degradation parameters (Table 6 and Figs. 4 and 5). This finding is reinforced by the lack of correlation between the lignin contents obtained by these methods and the gravimetric methods (Table 5).

Recent research on the evaluation of soluble lignin in acetyl bromide has raised the possibility of obtaining more accurate estimates of lignin content in feed by minimizing the interference by other compounds, mainly using the cell wall as a base in detriment to the acid detergent insoluble residue (Fukushima and Dehority, 2000; Fukushima and Hatifield, 2004; Fukushima et al., 2009).

In some studies, the correlations between the ABLcw contents and *in vitro* digestibility of DM or other cell wall components were stronger than were those obtained with Lignin (sa) or Lignin (pm) (Fukushima and Dehority, 2000; Fukushima and Hatifield, 2004). However, in these studies, the spectrophotometric standards were obtained independently for each evaluated material rather than using the universal standard curve proposed by Fukushima and Kerley (2011) and adopted in this study.

According to Fukushima and Dehority (2000), the lignin extracted from a forage sample cannot be utilized as a standard for the analysis of samples obtained from different species or forages at different stages of maturation. Empirical regression equations, like that proposed by Fukushima and Kerley (2011), are population-dependent. These models are based exclusively on experimental information rather than on a theoretical or biological basis. Therefore, even with good data adjustment, the model must be considered to be specific to the conditions in which the data were obtained, and its predictive value will be limited (Forbes and France, 1993).

Therefore, considering previous information (Fukushima and Dehority, 2000; Fukushima and Hatifield, 2004), the lack of association between the lignin contents determined by ABLadf and ABLcw may be related to the inefficiency of standard curve prediction suggested by Fukushima and Kerley (2011). This finding confirms that the evaluation of soluble lignin in acetyl bromide is restricted by the need to obtain specific standards for each type of material evaluated.

5. Conclusions

The lignin contents obtained by the Klason method, by cellulose solubilization in sulfuric acid and by oxidation with potassium permanganate present protein contamination. Therefore, protein correction is suggested, particularly for the Klason lignin method. Legumes produce more prominent protein contamination than do grasses.

We better established relationships between lignin and the parameters of ruminal degradation of neutral detergent fiber using the estimates produced by the Klason method and by oxidation in potassium permanganate.

Acknowledgments

The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG-PPM), and the INCT Ciência Animal for financial support.

References

Association of Official Analytical Chemistry (AOAC), 1990. Official Methods of Analysis, 15th ed. AOAC International, Arlington.

Chang, X.F., Chandra, R., Berleth, T., Beatson, R.P., 2008. Rapid, microscale, acetyl bromide-based method for high-throughput determination of lignin content in *Arabidopsis thaliana*. J. Agric. Food Chem. 56, 6825–6834.

Clipes, R.C., 2007. Evaluation of parameters to estimate the degradation potential of fibrous and nitrogenous compounds in tropical grasses. D.S. Thesis. Universidade Estadual do Norte Fluminense, Campos dos Goytacazes (in Portuguese with English abstract).

Detmann, E., Valadares Filho, S.C., 2010. On the estimation of non-fibrous carbohydrates in feeds and diets. Arq. Bras. Med. Vet. Zootec. 62, 980–984.

Detmann, E., Paulino, M.F., Valadares Filho, S.C., 2008. Avaliação nutricional de alimentos ou de dietas? Uma abordagem conceitual. In: Proceedings of 2nd International Symposium on Beef Cattle Production , Viçosa, Brazil, pp. 21–52.

Detmann, E., Paulino, M.F., Mantovani, H.C., Valadares Filho, S.C., Sampaio, C.B., Souza, M.A., Lazzarini, I., Detmann, K.S.C., 2009. Parameterization of ruminal fibre degradation in low-quality tropical forage using Michaelis–Menten kinetics. Liv. Sci. 126, 136–146.

Draper, N., Smith, H., 1966. Applied Regression Analysis. John Willey and Sons, New York.

Ellis, W.C., Matis, J.H., Hill, T.M., Murphy, M.R., 1994. Methodology for estimating digestion and passage kinetics of forages. In: Fahey Jr, G.C. (Ed.), Forage Quality, Evaluation, and Utilization. American Society of Agronomy, Madison, pp. 682–756.

Eskin, N.A.M., Henderson, H.M., Townsend, R.J., 1971. Biochemistry of Foods. Academic Press, New York.

Forbes, J.M., France, J., 1993. Introduction. In: Forbes, J.M., France, J. (Eds.), Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, Wallingford, pp. 1–12.

Fukushima, R.S., Dehority, B.A., 2000. Feasibility of using lignin isolated from forages by solubilization in acetyl bromide as a standard for lignin analyses. J. Anim. Sci. 78, 3135–3143.

Fukushima, R.S., Hatifield, R.D., 2001. Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. J. Agric. Food Chem. 49, 3133–3139.

Fukushima, R.S., Hatifield, R.D., 2004. Comparison of the acetyl bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. J. Agric. Food Chem. 52, 3713–3720.

Fukushima, R.S., Kerley, M.S., 2011. Use of lignin extracted from different plant sources as standards in the spectrophotometric acetyl bromide lignin method. J. Agric. Food Chem., doi:10.1021/jf104826n.

Fukushima, R.S., Savioli, N.M.F., 2001. Correlation between in vitro cell wall digestibility and three analytical methods for quantifying lignin. R. Bras. Zootec. 30, 302–309 (in Portuguese with English abstract).

Fukushima, R.S., Kerley, M.S., Porter, J.H., Kallenbach, R., 2009. The acetyl bromide lignin method to quantify lignin content in. In: Proceedings of 46th Meeting of Brazilian Society of Animal Science , Maringá, Brazil (eletronic proceedings; in Portuguese with English abstract).

Hatfield, R., Fukushima, R.S., 2005. Can lignin be accurately measured? Crop Sci. 45, 832–839.

Hatifield, R.D., Jung, H.G., Raplh, J., Buxton, D.R., Weimer, P.J., 1994. Comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. J. Agric. Food Chem. 65, 51–58.

Henriques, L.T., Detmann, E., Queiroz, A.C., Valadares Filho, S.C., Leão, M.I., Paulino, M.F., 2007. Fractions of cell wall nitrogenous compounds in tropical forages. Arq. Bras. Med. Vet. Zootec. 59, 258–263 (in Portuguese with English abstract).

Iiyama, K., Wallis, A.F., 1990. Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. J. Sci. Food Agric. 51, 145–161.

Johnson, D.B., Moore, W.E., Zank, L.C., 1961. The spectrophotometric determination of lignin in small wood samples. Tappi 44, 793–798.

Jung, H.G., Allen, M.S., 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73, 2774–2790.

Jung, H.G., Varel, V.H., 1988. Influence of forage type on ruminal bacterial populations and subsequent *in vitro* fiber digeston. J. Dairy Sci. 71, 1526–1535.

Jung, H.G., Vogel, K.P., 1986. Influence of lignin on digestibility of forage cell wall material. J. Anim. Sci. 62, 1703–1712.

Jung, H.G., Mertens, D.R., Payne, A.J., 1997. Correlation of acid detergent lignin and Klason lignin with digestibility of forage dry matter and neutral detergent fiber. J. Dairy Sci. 80, 1622–1628.

Kondo, T., Mizuno, K., Kato, T., 1987. Some characteristics of forage plant lignin. Jpn. Agric. Res. Quart. 21, 47–52.

Licitra, G., Hernandez, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347–358.

Lowry, J.B., Conlan, A.C., Shlink, A.C., Mcsweeney, C.S., 1994. Acid detergent dispersible lignin in tropical grasses. J. Sci. Food Agric. 65, 41–49.

McDougall, E.I., 1949. Studies on ruminal saliva. 1. The composition and output of sheep's saliva. J. Biochem. 43, 99–109.

Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85, 1217–1240.

Morrison, I.M., 1972. Improvements in the acetyl bromide technique to determine lignin and digestibility and its application to legumes. J. Sci. Food Agric. 23, 1463–1469.

Pell, A.N., Schofield, P., 1993. Computerized monitoring of gas production to measure forage digestion *in vitro*. J. Dairy Sci. 76, 1063–1073.

Popoff, T., Theander, O., 1976. Formation of aromatic compounds from carbohydrates. IV. Chromones from reaction of hexuronic acids in slightly acid, aqueous solution. Acta Chem. Scand. 30, 705–710.

Savioli, N.M., Fukushima, R.S., Lima, C.G., Gomide, C.A., 2000. Yield and spectrophotometric pattern of lignin extracted from cell wall, neutral detergent fiber or acid detergent fiber. R. Bras. Zootec. 29, 988–996 (in Portuguese with English abstract).

Theander, O., Westerlund, E.A., 1986. Studies on dietary fiber. 3. Improved procedures for analysis of dietary fiber. J. Agric. Food Chem. 34, 330–336.

Traxler, M.J., Fox, D.G., Van Soest, P.J., Pell, A.N., Lascano, C.E., Lanna, D.P.D., Moore, J.E., Lana, R.P., Vélez, M., Flores, A., 1998. Predicting forage indigestible NDF from lignin concentration. J. Anim. Sci. 76, 1469–1480.

Van Milgen, J., Murphy, L.L., Berger, L.L., 1991. A compartmental model to analyze ruminal digestion. J. Dairy Sci. 74, 2515–2529.

Van Soest, P.J., 1963. Symposium on nutrition and forage and pastures: new chemical procedures for evaluating forages. J. Anim. Sci. 22, 838–845.

Van Soest, P.J., 1967. Development of comprehensive system of feed analyses and application to forages. J. Anim. Sci. 26, 119–128.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, 2nd ed. Cornell University Press, Ithaca.

Van Soest, P.J., Robertson, J.B., 1985. Analysis of Forages and Fibrous Foods. Cornell University Press, Ithaca.

Van Soest, P.J., Wine, R.H., 1968. The determination of lignin and cellulose in acid-detergent fibre with permanganate. J. Assoc. Off. Anal. Chem. 51, 780–785.

Vieira, R.A.M., Pereira, J.C., Malafaia, P.A.M., Queiroz, A.C., 1997. The influence of elephant grass (*Pennisetum purpurem* Schum. Mineiro variety) growth on the nutrient kinetics in the rumen. Anim. Feed Sci. Technol. 66, 197–210.

Whitehead, D.L., Quicke, G.V., 1964. A comparison of six methods of estimating lignin in grass hay. J. Sci. Food Agric. 15, 417–422.

Whitmore, F.W., 1982. Lignin-protein complex in cell walls of *Pinus elliottii*: amino acid constituents. Phytochemistry 21, 315–318.